STED microscopy with a supercontinuum laser source

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Abstract: We report on a straightforward yet powerful implementation of stimulated emission depletion (STED) fluorescence microscopy providing subdiffraction resolution in the far-field. Utilizing the same supercontinuum pulsed laser source both for excitation and STED, this implementation of STED microscopy avoids elaborate preparations of laser pulses and conveniently provides multicolor imaging. Operating at pulse repetition rates around 1 MHz, it also affords reduced photobleaching rates by allowing the fluorophore to relax from excitable metastable dark states involved in photodegradation. The imaging of dense nanoparticles and of the microtubular network of mammalian cells evidences a spatial resolution of 30–50 nm in the focal plane, i.e. by a factor of 8–9 beyond the diffraction barrier.

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References and links

1. Introduction

Owing to its molecular specificity and notoriously simple sample preparation, far-field fluorescence microscopy has become one of the most commonly applied techniques in the life sciences. However, the focal plane resolution of all standard versions of far-field fluorescence microscopy is limited by diffraction to $\Delta x \approx \lambda / (2NA)$, with $\lambda$ denoting the wavelength of light and NA the numerical aperture of the objective lens. The advent of stimulated emission depletion (STED) microscopy [1-4] demonstrated that the limiting role of diffraction can be fundamentally overcome and that fluorescence microscopy with a resolution up to the macromolecular scale is possible with conventional optics. Meanwhile, far-field fluorescence nanoscopy has developed into a rapidly emerging field [5-8] having the potential to transform many areas of applications, in particular the life sciences.

The basic idea behind scanning STED microscopy is to confine the fluorescence emission of fluorophore markers to a region that is much smaller than the region covered by the diffraction-limited excitation spot. STED microscopy can be implemented with both continuous wave [9] and pulsed lasers, but a more efficient confinement has so far been achieved in the latter case [4]. In a typical implementation, a train of laser pulses capable of exciting the fluorophores is focused with an objective lens to a diffraction-limited spot which is overlapped with the focus of a pulsed ‘STED’ beam exhibiting a central zero-intensity point and steep intensity edges in the focal area. To improve the resolution in the focal plane the STED beam is usually formed as a doughnut that is coaligned with the excitation spot [3]. The pulses of the STED beam reach the focal plane virtually simultaneously with, or a few picoseconds after, the excitation pulses so as to instantly de-excite potentially excited molecules by stimulated emission. To this end, the wavelength of the STED laser is tuned to the red edge of the emission spectrum of the fluorophore. The pulses are most effective if their duration is a fraction of the 1–5 ns lifetime of the fluorescent state [3].

The probability of a dye molecule to emit decreases with the STED pulse intensity. As a result, the ability to fluoresce is confined to a subdiffraction-sized region around the doughnut center, defining the effective point-spread-function (PSF) of the microscope. Neighboring objects that are closer than the diffraction limit can be read out sequentially in time by scanning the beams across them. If one defines an intensity $I_s$ at which 50% of the fluorophores are quenched, the full-width-half-maximum (FWHM) of the effective PSF in the focal plane can be approximated by $\Delta x = \lambda \sqrt{2NA_0(1 + I_s/I_s)}[10, 11]$; for typical organic dyes $I_s$ amounts to 10–30 MW/cm².

Triplet-state or dark-state relaxation (T-Rex or D-Rex) STED microscopy is a highly effective variant of STED microscopy that has so far provided the highest resolution [4]. The basic idea behind this illumination scheme is to ensure that the time interval between consecutive pulse pairs is larger than the average lifetime of an excited metastable dark state (e.g. the triplet state) of the fluorophore. Since the average lifetime of such dark states is typically in the microsecond range, pulse repetition rates of <1 MHz inherently ensure that the dark states have relaxed by the time the subsequent pulse pair arrives. Because dark states (in particular, the triplet state) are known to be involved in photobleaching via charge tunneling [12, 13] or triplet absorption [14], T-Rex conditions reduce photobleaching of dyes having a
significant dark state build-up and, by the same token, enable larger ratios $I/I_s$ and hence smaller values $\Delta r$.

Unfortunately, implementing a T-Rex setup with tunable excitation and depletion wavelength has so far required a mode-locked, large-frame pumped Ti:sapphire oscillator pumping a regenerative amplifier (RegA) that in turn fed an optical parametric amplifier (OPA) offering the required range of visible wavelengths [4]. The cost of such a system easily amounts to half a million US dollars. Moreover, efficient nonlinear wavelength conversion required the oscillator and hence the OPA to deliver pulses of 200–300 fs duration. This is a drawback since STED is most effective with 0.1–2 ns pulses. To make up for the $3–4$ orders of magnitude difference in pulse duration, the pulses had to be stretched by dispersion using gratings or fibers which added to the complexity of the system. All this contributed to the notion that STED microscopy is inherently expensive, requires elaborate instrumentation, as well as diligent operation and maintenance. In this paper, we demonstrate that recent advances in photonic-crystal fiber-based supercontinuum generation [15] enable a straightforward, economic, and rugged implementation of T-Rex STED microscopy that should greatly facilitate the wider dissemination of this all-optical method of improving the spatial resolution in far-field fluorescence microscopy.

Recently, compact supercontinuum systems have emerged, based on a mode-locked fiber oscillator, fiber amplifier, and a photonic crystal fiber generating a broadband spectrum by a number of nonlinear effects, among them self-phase modulation, stimulated Raman scattering and frequency mixing [16]. The pulse energies generated by the first generation supercontinuum sources, including those pumped by 76 MHz mode-locked Ti:sapphire lasers, proved very useful as excitation pulses in STED microscopy [17]. However, they were too low in power for STED. Fortunately, more recent systems provide spectral power densities exceeding 1 mW/nm at a repetition rate of 1 MHz in the visible range $>630$ nm. For most fluorophores, the useful spectral bandwidth of a STED pulse typically spans over 20 nm which allows the selection of STED pulses of 20 nJ using appropriate filters. These pulse energies are comparable to or even higher than those of the lasers commonly reported in STED microscopy. In conjunction with strong focusing to typical doughnut areas of the order $10^{-9}$ cm$^2$, they readily provide the required intensities $I$ of several GW/ cm$^2$ in the focal region, even after the inevitable losses in the optical pathway. In the range 470–630 nm, the pulse energy of these systems is still too low for STED, but abundant for the excitation of fluorophores. Due to the fact that STED is performed at the red edge of the emission spectrum of the dye, these sources should allow STED microscopy of dyes having fluorescence peaks at wavelengths $>580$ nm, i.e. of yellow, red and far-red emitting fluorophores.

2. Setup

The STED microscope sketched in Fig. 1 employs a customized version of a commercial supercontinuum (SC) fiber laser providing both the excitation and the STED pulses (SC-450 HP, Fianium, Southampton, UK) at about a tenth of the cost of a typical laser system used for T-Rex illumination and with a shoebox-sized footprint. While the master oscillator of the system is specified to provide 5–10 ps pulses, the pulse length of a 20 nm spectral band of the supercontinuum was measured to be $(82 \pm 10)$ ps as determined by time-correlated single-photon counting (TCSPC) using a microchannel plate detector and considering the width of the instrument response function (IRF) of $\sim 35$ ps. The pulses are therefore already appropriate for STED and do not require any further stretching. While originating from the same resonator, the excitation and STED pulses are inherently synchronized and their simultaneous arrival at the focal plane can be adjusted by equalizing the optical path lengths with a comfortable tolerance of $\pm 0.5$ cm. Thus, apart from the laser source and the optical components typically required for a scanning (confocal) microscope, this STED microscope design necessitates only few additional components.
Fig. 1. Experimental Setup. The randomly polarized laser beam is split into two beams using a polarizing beam splitter cube (PB) from which the excitation and STED wavelengths are extracted by means of an interference filter (EF) and a prism-based wavelength selector (WS), respectively. Both beams are spatially filtered by coupling them into single-mode fibers (SMF), expanded and coupled into a confocal setup using two dichroic beamsplitters (D1, D2). The focal doughnut is created by passing the STED beam through a vortex phase mask (PM). DF: detection filter, MMF: multimode fiber; APD: avalanche photodiode; P: prism; S: slit.

First, the infrared part ($\lambda > 950\text{ nm}$) of the randomly polarized supercontinuum was stripped from the visible spectrum with two dielectric mirrors (DLB 350–950 nm, LINOS Photonics AG, Göttingen, Germany). Two orthogonally polarized but otherwise equivalent beams were established using a polarizing beam splitter cube; one was used for excitation whereas the other one for STED. The use of a polarizing beam-splitter ensured a defined polarization in each beam which was useful for establishing a well-defined polarization state in the focal region.

A convenient way to extract the required excitation and STED wavelengths from the supercontinuum is to use interference bandpass filters. We chose this implementation for the excitation beam but not for the STED beam where care had to be exerted to reject the wavelengths outside the desired ~20 nm range. The requirement for a sharp passband for the STED pulses stems from the fact that for a typical organic fluorophore, STED is effective within a ~20 nm wavelength range of the red tail of its emission spectrum. Therefore the beam meant for STED was coupled into a wavelength selector (see inset of Fig. 1) built around a highly dispersive Brewster prism (SF59, Schott AG, Mainz, Germany) which allowed a compact design. The 4f arrangement guarantees that all colors come back onto one beam. Therefore no alignment is necessary after a wavelength change. The slit is positioned nearly at the focus of the lens ($f = 120\text{ mm}$, Linos AG, Göttingen, Germany) which provides very steep edges of the filter. By simply changing the position and the width of a mechanical slit placed in the expanded supercontinuum spectrum, the center wavelength and the spectral width of the STED beam could be freely adjusted. It was thus possible to optimize the STED spectrum for the respective fluorophore even while the measurement was running.

Both beams were separately coupled into polarization-maintaining single mode optical fibers. The fiber outputs were collimated and colinearly coupled into an oil immersion objective lens (PL APO 100x/1.40–0.7 Oil, Leica Microsystems, Wetzlar, Germany) using two dichroic mirrors (D1 and D2). The fluorescence was collected by the same objective lens and separated from the laser light by means of D1 and D2 and an additional bandpass filter (DF). The particular filter combinations for the employed excitation (532 nm, 570 nm, 630 nm) and STED wavelengths (650 nm, 700 nm, 745 nm) are listed in Table 1. The
collected fluorescence was focused into a multimode optical fiber (62.5 µm / 0.27 NA, M31L01, Thorlabs) which acted as a confocal pinhole of 1.12–1.40 times the size of an Airy disc. The fluorescence photons were registered with an avalanche photodiode module (SPCM-AQRH-13-FC, Perkin Elmer, Vaudreuil, Québec, Canada) connected to a time-correlated single-photon counting board (SPC-830, Becker & Hickl GmbH, Berlin, Germany). The image acquisition was performed by scanning the sample with a 3D piezo stage (NanoBlock, Melles Griot GmbH, Bensheim, Germany). Typically, images of 10 µm × 10 µm were acquired with a 20 nm pixel size (typically 512 × 512 pixels) and dwell times of 0.5–1.5 ms. The STED and confocal reference images were recorded nearly simultaneously on a line-by-line basis by opening and closing a shutter in the STED beam.

Table 1. Filter combinations used at different STED wavelengths.

<table>
<thead>
<tr>
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<th>(\lambda_{\text{STED}} = 650 \text{ nm})</th>
<th>(\lambda_{\text{STED}} = 700 \text{ nm})</th>
<th>(\lambda_{\text{STED}} = 750 \text{ nm})</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Excitation filter</strong></td>
<td>Z532/10X(^1)</td>
<td>Z570/10X(^1)</td>
<td>Z630/10X(^1)</td>
</tr>
<tr>
<td><strong>Optical fiber (excitation)</strong></td>
<td>PM S460-HP(^2)</td>
<td>PM S460-HP(^2)</td>
<td>PM630HP(^2)</td>
</tr>
<tr>
<td><strong>Optical fiber (STED)</strong></td>
<td>PMC-630(^3)</td>
<td>PMC-630(^3)</td>
<td>PMC-780(^3)</td>
</tr>
<tr>
<td><strong>Dichroic D1</strong></td>
<td>Z530RDC(^1)</td>
<td>Z568RDC(^1)</td>
<td>Z635RDC(^1)</td>
</tr>
<tr>
<td><strong>Dichroic D2</strong></td>
<td>HR660HT-UV(^4)</td>
<td>Z695SPRDC(^1)</td>
<td>Z710SPRDC(^1)</td>
</tr>
<tr>
<td><strong>Detection filter</strong></td>
<td>2x 582/75(^5)</td>
<td>HQ625/60(^6)</td>
<td>Z680/60M(^6)</td>
</tr>
</tbody>
</table>

\(^1\) AHF Analysentechnik GmbH, Tübingen, Germany.
\(^2\) Thorlabs GmbH Europe, Dachau, Germany.
\(^3\) Schäfer und Kirchhoff GmbH, Hamburg, Germany.
\(^4\) Laser Components GmbH, Olching, Germany.
\(^5\) Semrock, Inc., Rochester, NY, USA.

To create a focal doughnut, the expanded STED beam first passed through an achromatic half-wave plate (500–900 nm, B. Halle Nachf. GmbH, Berlin, Germany) to fine-tune the polarization orientation, and subsequently through a vortex phase plate \([18]\) (RPC Photonics, Rochester, NY, USA) imprinting a helical phase ramp of \(2\pi\) onto the wavefront. An achromatic quarterwave plate (500–900 nm, B. Halle GmbH) placed close to the back aperture of the objective lens afforded circular polarization of both the excitation and the STED beams which minimizes photoselection effects. Also, circular polarization of the STED beam causes all vectorial components of the light field to interfere destructively at the geometric focal point, thus creating a deep doughnut minimum.

3. Results

Unlike in previous STED experiments where the spectral width of the STED beam was determined by the type of laser employed, the prism selector allowed free selection of the center wavelength and of the width of the STED spectrum. Broadening the spectral width and thus the STED pulse energy is expected to yield larger ratios \(I_1/I_0\) and thus a higher spatial resolution. However, this strategy is limited by the useful bandwidth (~ 20 nm) of the STED light and the requirement for destructive interference in the center of the doughnut; the broader the STED spectrum, the less deep is the doughnut minimum. To determine the depth of the central minimum of the doughnut, we probed the doughnut by scanning gold nanoparticles (Ø 80 nm, EMGC80, BBInternational, Cardiff, UK) through the focal region, while registering the scattered light. Due to the inevitable background in the measurement, the central minimum can be determined down to a depth of ~1 % of the doughnut crest, which is also an acceptable upper limit for the depth. We found that the utilized vortex phase plate tolerates a spectral bandwidth up to approx. ~20 nm without exceeding this value.
We first tested our setup with samples of fluorescently stained polystyrene nanospheres (FluoSpheres® orange / red / crimson, Ø 40 nm, Invitrogen GmbH, Karlsruhe, Germany) mounted on a microscope coverslip and embedded in the mounting medium mowiol [19]. The imaging was performed at excitation / STED wavelength pairs of 532 / 650 nm, 570 / 700 nm and 630 / 745 nm. The pulse energies at the back aperture of the objective lens were ~1 pJ for the excitation and 1.4–2.4 nJ for the STED beam; the actual values in the focal region are reduced by a factor of 0.6–0.8, depending on the transmission of the objective lens at the particular wavelength.

Figure 2 shows side-by-side comparison images recorded in the confocal and the T-Rex STED mode. Closely spaced nanospheres are not resolved in the confocal reference images but are clearly discernible in the STED recordings. Line profiles across the beads indicate FWHM in the 50–60 nm range. They are, however, broadened by the 40 nm bead diameter. Taking into account this diameter reveals an all-optical resolution of 30–40 nm in the focal plane.

![Fig. 2. Comparison between confocal (middle) and STED images (left) of randomly dispersed 40 nm fluorescent beads at the indicated wavelengths. The upper, center, and lower row show the data of red, crimson, and orange beads, respectively. Postprocessing the raw data (left) by a Richardson-Lucy deconvolution algorithm further enhances the details (STED+, right). The line profiles along the traces indicated by blue and purple arrows demonstrate that full-width-at-half-maximum values between 49 nm and 58 nm were achieved in the raw data. Scale bar: 500 nm.](image)

To improve the contrast and the resolution further, we also applied a positivity-constrained, iterative image restoration (Richardson-Lucy) algorithm using the effective PSF estimated from the above recordings. The results, shown in Fig. 2, evidence a dramatic increase of resolution in the STED images. As can also be seen, the images show some fluctuations in the fluorescence signal as evidenced by a stripe-like pattern present in the confocal images but less so in the STED images. While the origin of these fluctuations has not
been systematically studied, we think that they are either induced by a transient population of the dark states or simply due to instabilities in the fiber coupling.

In Fig. 3 we imaged the tubular network of mammalian PtK2 cells which were stained using an immunofluorescence protocol involving a primary antibody (anti beta-Tubulin mouse IgG (monoclonal), Sigma, Saint Louis, USA) and a labeled secondary antibody (Sheep anti-mouse IgG, Dianova, Hamburg coupled to ATTO 565 / ATTO 590 / ATTO 633, ATTO-TEC GmbH, Siegen, Germany). These dyes are customary organic dyes; none of them is specifically optimized for STED microscopy.

Fig. 3. Immunolabeled tubulin fibers imaged with an excitation wavelength of 570 nm (top), 630 nm (middle), and 532 nm (bottom). The comparison between the confocal reference image (left) and the STED image (right) reveals the gain in structural information obtained by STED; note that all images represent raw data. The line profiles along the traces indicated by the blue and purple arrows highlights details in the STED image (purple) that are not discerned by the confocal microscope (blue). Scale bar: 1 µm.

The resolution enhancement became evident at all three wavelengths and dyes (see Fig. 3). While they are completely blurred in the confocal image, delicate structures and closely spaced fibers are revealed in the T-Rex STED image. Line profiles across the tubulin images revealed FWHM in the range of 60–80 nm. The diameters of a tubulin fiber plus the attached primary and secondary antibodies amount to a total of ~40–60 nm. Thus the actual resolution
of the STED microscope in these images also ranges between 30–50 nm. Note that the images represent raw data.

4. Discussion and conclusion

Our results demonstrate that (T-Rex) STED microscopy and hence a far-field fluorescence nanoscopy based solely on physical phenomena, can be realized with a single turn-key supercontinuum laser system. The demonstrated gain in resolution by up to a factor of 8–9 over the diffraction barrier is adequate for many applications, especially when considering that only half of the pulse energy emanating from the (randomly polarized) laser source was available for STED. In the future, the other half of the beam, used here to extract the excitation wavelength, could be employed to simultaneously enhance the resolution along the optic (z) axis. Alternatively, this beam could be utilized to set up another color channel.

We expect that further improvements in supercontinuum generation will also expand the range of applicability to blue and green emitting dyes. Nonetheless, we note that the supercontinuum source utilized covers a wavelengths range (650–750 nm) for STED, which is not covered by other pulsed laser sources; in fact, STED at 700 nm wavelength has so far not been reported. The viability of the organic fluorophore ATTO 590 for STED at 700 nm provides further evidence that for each visible wavelength, suitable organic fluorophores can be found for STED. This finding is arguably not surprising, since stimulated emission is a basic photophysical phenomenon of any (organic) fluorophore. While the usability of a fluorophore is ultimately limited by its photobleaching rate, the majority of fluorescent dyes can be employed for (T-Rex) STED microscopy.

In our experiments, multicolor imaging required the change of dichroic beamsplitters. Future improvements will take advantage of the fact that the supercontinuum source provides a large spectrum of wavelengths at the same time. Replacing the dichroic mirrors by acousto-optical filters or similar devices will increase the flexibility in wavelengths applied. The use of an achromatic vortex lens [20] avoids the exchange of phase filters.

The pulse lengths of the excitation and STED beams were almost equal, which to some extent results in undesirable excitation of fluorophores while the STED pulse is effective. Hence, a further improvement is expected from increasing the duration of the STED pulses, e.g. by stretching the pulses with a dispersive element such as a fiber.

The commercially available supercontinuum laser system operates at repetition rates of 1-5 MHz. While the ~1 µs time interval between subsequent pulses allows for efficient relaxation of fluorophores which have been transiently crossed to a dark state [4], the low repetition rate comes at the cost of long image acquisition times. In the current setup, the photon count rate was also limited to 1 MHz due to the ~30 ns dead time of the detector. With an adapted detection scheme (e.g. a multi-detector setup), a stronger fluorescence signal, realized by augmenting the excitation intensity, should result in reduced pixel dwell times. In conjunction with a fast scanning scheme, T-Rex conditions should be achievable even for a higher total flux.

In conclusion, STED fluorescence microscopy can be implemented with presently available supercontinuum laser sources. In conjunction with the attained all-optical resolution of 30–50 nm in the far-field, the versatility of the optical arrangement and the low equipment cost should facilitate the widespread adoption of this far-field fluorescence nanoscopy technique both for further development and its application.

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